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Full Length Research Paper

Development of somaclones in sugarcane genotype BF-162 and assessment of variability by random amplified polymorphic DNA (RAPD) and simple sequence repeats (SSR) markers in selected red rot resistant somaclones

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Worldwide, sugarcane (*Saccharum officinarum L*) is the major source of commercial sugar along with many other value added products. In Pakistan, during the year 2008 to 2009, there was a production of 50.05 million tonnes. Sugarcane genotype BF-162 was released for general use in the Punjab province during 1990, and it became susceptible to red rot. As environmental conditions are not conducive for flowering, so the red rot rectification was tried through somaclonal variation. Protocol for callogenesis and organogenesis was standardized. Leaf when used as explant source and 2,4-dichlorophenoxyacetic acid (2,4-D) as auxin at 3mg/l performed better in callogenesis. It was observed that lower doses of Kinetin regenerated more numbers of shoots, while indole-3-butyric acid (IBA) developed more numbers of roots. Red rot resistance somaclones were isolated and assessed for the presence of variability through random amplified polymorphic DNA (RAPD) and simple sequence repeats (SSR) markers. Polymorphism captured by RAPD was 33.73% and by SSR was 64%. Polymorphic information content (PIC) value ranged between 0.02 and 0.45 for RAPD and 0.12 and 0.49 for SSR. Cluster and sub cluster formation further verified the presence of variability in the red rot resistant somaclones with respect to the parent.

Key words: Sugarcane, callogenesis, organogenesis, somaclone, polymorphism, cluster.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L) is a major source of commercial sugar and has now emerged as a multipurpose crop providing not only sugar, but also a series of value added products and by-product. It is the second largest cash crop of Pakistan. In the year 2008 to2009, a production of 50.05 million tonnes was recorded for it (Anonymous, 2008). This signifies the need of continuous breeding efforts for the evolution of high yielding and disease resistance cultivars.

Sugarcane variety “BF-162” was released for general cultivation in Punjab province during the year 1990. In subsequent years, it became susceptible to red rot, leaf rust and Pokkah boeng. Conventional means did not allow its improvement as the agro climatic conditions are not conducive for flowering.

Red rot (*Colletotrichum falcatum* Went.) disease is the most dreadful disease of the region. During the years 2003 to 2006, it causes the greatest loss to sugarcane industry. There was 29.07% loss in cane weight and consequently 30.8% loss in sugar recovery (Hussnain

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; **IBA,** indole-3-butyric acid; **RAPD,** random amplified polymorphic DNA; **SSR,** simple sequence repeats; **PIC,** polymorphic information content.

and Afghan, 2006).

Somaclonal variation has been used by various scientists to recover improved plantlets from a number of genotypes. Somaclones may show variation for different parameters like yield, sugar recovery, disease resistance, drought tolerance and maturity. It is not controversial that tissue culture techniques are playing their part in sugarcane improvement, and at the same time for a plant breeder assessment of genetic diversity, they are very essential in tissue culture derived plants. This helps the breeder to select appropriate genetic material for running a breeding programme.

Genetic markers have contributed much to understanding plant genetic diversity. Molecular markers are extensively being used to measure the variability present at genetic level, within and among the genotypes. The most commonly applied molecular markers used to study polymorphism are random amplified polymorphic DNA (RAPD) and simple sequence repeats (SSR) (Nair et al., 1999, 2002; Oropeza et al.,1995). SSRs are more precise and highly polymorphic than other marker techniques such as restriction fragment length polymorphism (RFLPs) and RAPDs (Powell et al., 1996), and are even effective to measure variability between closely related lines.

Development of somaclones from any genotype requires optimum conditions for callus formation and its regeneration to get plantlets from an explant source. The objectives of this study have been planned to standardize the protocols for two explant sources in order to develop somaclones as well as to calculate the genetic variability in the selected red rot resistant somaclones by the use of DNA markers such as RAPD and SSR. These informations certainly will facilitate the supplementation of the ongoing breeding programme prevailing in Pakistan.

MATERIALS AND METHODS

The innermost young leaves above the apical meristem and pith explants below the meristem, both having a size of 0.5 mm were cultured on Murashige and Skoog (MS) medium (1962) fortified with 2,4 dichlorophenoxy acetic acid (2,4-D) and indole acetic acid (IAA) at 1,3,5,7 & 9 mg/l for callogenesis. Calli developed in each treatment were separately cultured on MS medium having Kinetin at 0.1, 0.5, 1.0, 1.5 and 2.0 mg/l along with casine hydrolysate at 480 mg/l for organogenesis. Differentiated shoots were singled out and shifted to the rooting medium containing 1/2 or full concentration of MS salts with five different concentrations, that is, 0.1, 0.5, 1.0, 1.5 and 2.0 mg/l of IBA and NAA separately. Ten cultures for each treatment both for callus induction and differentiation were practiced.

Initially, callus cultures were kept under dark to avoid phenolic substances for 15 days. Cultures for both callogenesis and organogenesis were put at 28±1°C temperature and for 16 h illumination between 2000 and 3000 lux intensity. Observations were recorded on callus induction and on the degree of callus formation, that is, 0 for no; + (1) for poor; ++ (2) for fair; +++ (3) for good and ++++ (4) for excellent callus. The first observation was recorded at the completion of the dark period and weekly after that. Observations regarding regeneration percentage and number of shoots obtained for each treatment were recorded after 40 days of culture, whereas those for root initiation and number of roots developed by each plantlet were recorded after 30 days.

Four weeks old rooted plantlets were removed from the culture tubes and transferred to plastic bags (12 x 20 cm in size) containing garden and loamy soils (3:1 v/v). Plants were watered immediately and kept under shade till the commencement of the sowing season. Plantlets were hardened under green house conditions and transferred to the experimental field of Agricultural Biotechnology Research Institute (AARI), Faisalabad, Pakistan at a row-to-row and plant to plant distance of 1.10 and 1.0 m, respectively in order to grow R_0 along with the donor.

Field growing somaclones in R_0 generation were screened out for red rot resistance using the borehole method. Three canes from each somaclone were artificially inoculated by the pathotype isolated from the donor plant separately on the middle of the third exposed internode from bottom during the 2nd week of July. Each millilitre of this spore suspension contained 1 x 10^6 spores. One millilitre of spore suspension was injected into the bore holes of each cane. After 60 days of inoculation, in the month of September, the red rot reaction was measured based on a 0-9 scale (Srinivasan and Bhatt, 1961). This was repeated in R_1 and R_2 generations also.

For molecular studies, young leaves from 15 red rot resistant somaclones (BF-162-01 to BF-162-15) and their donor parent (BF-162-P) were taken from R_2 generation and grinded in liquid nitrogen to extract DNA. Entries used are subsequently listed.

DNA was extracted by adopting the standard CTAB method (Doyle and Doyle, 1990). The DNA concentration was determined by Nano drop Spectrophotometer (ND 1000) and was diluted to a concentration of 15 ng/µl. Samples were stored at -20°C for further use. Polymorphism was studied using random amplified polymorphic DNA (RAPD) as illustrated by Williams et al. (1990) and simple sequence repeats (SSR) as described by Powell et al. (1996). Out of the 100 RAPD primers (GeneLink, USA) and 50 SSR primers used, 20 and 28 polymorphic primers were found respectively. List of polymorphic primers and their sequences are subsequently given.

The reaction mixtures (25 µl for RAPD and 20 µl for SSR studies) were amplified for each DNA sample in a Thermal Cycler (Eppendorf DNA thermal cycler 9600). Agarose gel (1.2%, w/v) was used for RAPD electrophoresis, whereas 3% (w/v) metaphor gel was used for SSR electrophoresis. The Ethidium Bromide at 100 µg/ml was added in jell for detection. Bands were viewed under ultraviolet trans-illuminator and “Syngene G. Box” documentation, and analysis system was used to make photographs.

The presence and absence of a DNA fragment was considered as basis of polymorphism. DNA loci if present were scored as ‘1’, and if not were scored as ‘0’. The number of alleles per locus was determined and the Polymorphic information content (PIC) values were calculated using the formula, as suggested by Cordeiro and Henry, (2001). However, monomorphic fragments were not considered. Coefficient of similarity was calculated by Nei and Li (1979) using NTSYSpc (version 2.1) cluster analysis software (Exeter Software Co., Setauket, NY). A dendrogram was constructed by using Unweighted Pair Group Method of Arithmetic means (UPGMA) algorithm (Sneath and Sokal, 1973) provided in the software package NTSYSpc (Rohlf, 1993).

RESULTS AND DISCUSSION

Callogenesis

A wide range of variation in callus texture, callus colour and degree of callus formation was examined with respect to culture media formulations, explant source

Table 1. Response to callogenesis while using 2,4-dichlorophenoxy acetic acid (2,4-D) and indole acetic acid (IAA) in Murashige and Skoog (MS) medium (162).

Hormone concentration (mg/l)	MS+2,4-D				MS+IAA			
	Callus Induction (%)		Callus Formation		Callus Induction (%)		Callus Formation	
	Leaf	Pith	Leaf	Pith	Leaf	Pith	Leaf	Pith
1.00	**96.66**	76.66	3.23±0.23	1.95±0.26	70.00	63.33	1.30±0.11	0.68±0.29
3.00	**96.66**	**86.66**	**3.61±0.34**	**2.68±0.21**	73.33	73.33	1.60±0.07	1.05±0.14
5.00	93.33	76.66	2.63±0.42	1.43±0.38	73.33	76.67	1.85±0.38	1.20±0.35
7.00	86.66	73.33	1.95±0.27	1.25±0.18	**90.00**	80.00	**2.56±0.33**	**2.08±0.26**
9.00	83.33	**63.33**	1.60±0.38	1.00±0.39	86.67	**86.67**	2.43±0.18	1.60±0.32
Leaf Mean	91.33		**2.60±0.38**		78.67		1.94±0.24	
Pith Mean	75.33		1.66±0.30		**76.00**		1.32±0.24	
Variety Mean	83.33		**2.13 ± 0.28**		77.33		**1.64±0.23**	

and genotype. Various researchers also observed such highly diversified mixture of calli formed in sugarcane cultures as reported by Brisbe et al. (1994), Falco et al. (1996) and Anbalagan et al. (2000). Anbalagan et al. (2000) observed two types of calli from leaf explants; one was loose, friable and non-embryogenic (non-regenerable) and the other was compact, white, nodular and embryogenic (regenerable). Callus texture was so heterogeneous that lots of confusions exist as regards its nomenclature. In this study, two prominent callus types, that is, compact (embryogenic) and friable (nonembryogenic) were observed.

Callogenesis response using 2,4-D in MS medium

In order to evaluate the effect of 2,4-D on callus induction and formation, the results were recorded for leaf and pith cultured on MS medium supplemented with its different concentrations. At low concentrations of 2,4-Dichlorophenoxy acetic acid (2,4-D), light green yellowish embryogenic callus from leaf and pith explants was observed. The highest degree (3.61±0.34) of callus formation was recorded from the young leaf explant cultured on MS medium supplemented with 3.0 mg/l 2,4-D (Table 1).

Pith explant also responded good in 3 mg/l, 2,4-D with the callus scale of 2.68±0.21, followed by 1 mg/l 2,4-D at both explants. Results indicate that lower levels of 2,4-D had a good callus than the higher concentrations. With the increase of hormone level, the callus formation declined. The results are in line with the findings of Michael (2007) and Khan et al. (2008, 2009).

The extent of callus induction was assessed as the percentage of the total number of explants forming callus. The callus initiation was recorded after 15 days of culturing explants. Callus initiation took place at all levels of 2,4-D in both the explants; however, it varied with the media used and explant source, ranging between 70.00 and 96.66% from leaf explant and between 63.33 and 86.66% from pith explant. The highest callus induction (96.66%) was noted on leaf explants cultured on 1.00 and 3.00 mg/l.

Callogenesis response using IAA in MS medium

Effect of IAA on callus induction and formation was also studied. It produced light yellowish and embryogenic calli. Leaf explant responded the best at 7 mg/l (2.56±0.33), followed by 9 mg/l (2.43±0.18). On decreasing the IAA concentration, the degree of callus formation was significantly reduced. At 1 mg/l concentration, the minimum degree of callus formation (1.30±0.11) was recorded. Pith explant produced maximum callus (2.08±0.26) at 7 mg/l concentration, followed by 1.60±0.32 degree of callus formation at 9 mg/l, and the least responsive (0.68±0.29) was 1 mg/l IAA concentration.

It is obvious from the findings that higher levels of IAA proved better than the lower concentrations. With the increase of hormone, more and more amount of calli was formed. Wen et al. (1991) also reported the same findings that the higher concentration of IAA in the media produced more amount of calli.

Rate of callus initiation from two explant types on different concentrations of IAA were also investigated. It indicated that leaf explant had the best response with 90% callus induction at 7 mg/l IAA concentration callus. This was followed by 9 mg/l with 86.67% callus induction. At 1 mg/l concentration, minimum callus initiation (70%) was recorded. By pith explant, maximum (86.67%) cultures developed calli at 9 mg/l, followed by callus induction (80%) at 7 mg/l IAA. However, the minimum callus induction (63.33%) was recorded at 1 mg/l.

The data obtained from this study confirmed the superiority of leaf explant over pith explant and the superiority of 2, 4-D as auxin over IAA for callus formation. Leaf as explant source emerged as the best callus producer over pith. On the whole, IAA produced1.64±0.23 degree of callus formation which is below that of callus formation

Table 2. Response to organogenesis while using calli developed on MS medium containing 2,4-D or IAA separately.

MS+ Kinetin (mg/l)	Calli developed by using 2,4-D as auxin				Calli developed by using IAA as auxin			
	Leaf explant		Pith explant		Leaf explant		Pith explant	
	Regeneration %[Y]	Average number of shoot	Regeneration %[Y]	Average number of shoot	Regeneration %[Y]	Average number of shoot	Regeneration %[Y]	Average number of shoot
0.1	80	07.30 ± 0.52	60	3.16 ± 0.23	70	5.30 ± 0.44	50	2.76 ± 0.21
0.5	90	11.03 ± 0.72	70	4.03 ± 0.37	**90**	**5.73 ± 0.39**	50	2.96 ± 0.14
1.0	**100**	**29.63 ± 0.84**	**80**	**6.76 ± 0.39**	**90**	4.56 ± 0.31	**60**	**3.30 ± 0.09**
1.5	**100**	19.83 ± 0.73	70	6.30 ± 0.32	80	4.23 ± 0.23	40	2.43 ± 0.18
2.0	90	08.48 ± 0.52	40	2.44 ± 0.45	70	3.53 ± 0.75	30	1.75 ± 0.63
Mean	92	15.25	64	4.53	80	4.67	46	2.64
Mean Square		102.16**		9.06**		1.39***		0.39***

when 2,4-D (2.13 ± 0.28) was used as auxin. This might be due to higher response of 2,4-D, which inspired a number of workers (Mannan and Amin, 1999; Prajapati et al., 2000; Hanafy et al., 2007) to use it.

Pith was found less responsive to callogenesis, which might be due to the production of polyphenols in the medium when pith was used as explant. Dark incubation reduces the secretion of polyphenol, resulting to induction and proliferation of calli at the initial stage. This is in accordance with the findings reported earlier by Shahid et al. (1994) and with those of Aftab and Iqbal (1999) and Snyman et al. (2001).

Organogenesis: Regeneration of micro shoots

Leaf source calli produced on MS medium having 2,4-D, when cultured on MS medium supplemented with 1.0 mg/l kinetin had the best performance. At this concentration, 100% of cultures produced shoots. The number of usable shoots was 29.63 ± 0.84. It was followed by the MS medium supplemented with 1.5 mg/l of kinetin in which the average number of usable shoots produced was 19.83 ± 0.730 (Table 2). The minimum number of shoots (07.30 ± 0.52) was produced by the medium supplemented by 0.1 mg/l of kinetin. Calli formed by pith explant on MS+2,4-D media, when cultured on regeneration medium produced 6.76 ± 0.39 usable shoots on MS medium supplemented with 1.0 mg/l kinetin (Table 2) and 80% cultures initiated shoot emergence. It was followed by MS medium having 1.5 mg/l of kinetin, which produced 6.30 ± 0.32 average numbers of usable shoots with 70% cultures initiated organogenesis.

MS medium supplemented with 0.5 mg/l kinetin had the best performance when calli produced by leaf explant and cultured on MS medium having IAA, were used (Table 2). At this concentration, 90% of cultures produced shoots.

The number of usable shoots was 5.73 ± 0.39, followed by MS medium supplemented with 0.1 mg/l of kinetin in which the average number of produced usable shoots was 5.30 ± 0.44 and 70% cultures showed regeneration capacity. Minimum number of shoots (3.53 ± 0.75) was produced by the medium supplemented by 2.0 mg/l of kinetin with 70% regeneration response by the calli cultured.

Calli produced by pith explant on MS medium having IAA were also used to study the regeneration capacity. The best performance was showed on MS medium supplemented with 1.0 mg/l kinetin. At this concentration, 60% of cultures produced shoots and the number of usable shoots was 3.30 ± 0.09. It was followed by MS medium supplemented with 0.5 mg/l of kinetin in which the average number of usable shoots produced was 2.96 ± 0.14 and 50% cultures showed regeneration capacity (Table 2). Minimum number of shoots (1.75 ± 0.63) was produced by the medium supplemented by 2.0 mg/l of kinetin with 30% regeneration response by the calli cultured. Results reported in the foregoing indicate that the low level of cytokinin/kinetin was more responsive to regeneration, which are in line with the findings of Javid et al. (2001), Ali et al. (2008) and Ather et al. (2009).

Effect of explant is evident from the data given in Table 2. Results depict that leaf source calli produced more than twice the number of shoots produced by pith source calli. This indicates that leaf source calli are more meristematic and certainly more morphogenic than pith source calli. This is in line with previous findings of Shahid et

Table 3. *In vitro* induction of roots in MS and ½MS medium supplemented with various concentrations of indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA)[X].(162).

Concentration (mg/l)	Full MS		½MS	
	Response (%)[Y]	Average number of root[Z]	Response (%)[Y]	Average number of root[Z]
Root development in MS and ½MS medium supplemented by IBA				
0.5	30	2.27 ± 0.26	40	3.50± 0.49
1.0	50	4.75± 0.57	55	3.85 ± 0.53
1.5	**60**	**5.35 ± 0.63**	60	4.75 ± 0.41
2.0	35	5.00 ± 0.52	**75**	**8.25 ± 0.64**
2.5	35	4.25 ± 0.59	50	5.05 ± 0.25
Average	42	4.32	56	5.08
Root development in MS and ½MS medium supplemented by NAA				
0.5	25	2.72 ± 0.35	30	2.45± 0.34
1.0	35	3.25 ± 0.46	45	3.38 ± 0.45
1.5	**55**	**4.31 ± 0.63**	55	4.57 ± 0.44
2.0	35	4.05 ± 0.51	**70**	**6.50 ± 0.56**
2.5	20	3.35 ± 0.42	35	4.89 ± 0.32
Average	34	3.53	47	4.35

[X]Rated after 30 days of culture; [Y]Data are %age of 20 replications; [Z]Mean of 20 replication ± standard error.

al. (1990) and Rehman et al. (2005).Average number of shoots produced while using 2,4-D as auxin was recorded as 15.25 and 4.53 from leaf and pith respectively whereas for IAA, it was 4.67 and 2.64. It also indicates that 2,4-D as auxin produced calli that had a better response to regeneration as compared to calli produced while using IAA as auxin. It can also be suggested that callus tissue cells produced in 2,4-D were more receptive to kinetin for shoot formation. It can be concluded from the aforementioned results that callogenesis and organogenesis potential was specific to explant source, genotype used and concentration to hormone used (Maretzki and Nickell, 1973; Maretzki, 1987). It was also observed that callus derived from different auxins showed different regeneration potentials. Nonetheless, callus induction, proliferation and regeneration potential seem to be interalated.

All five concentrations of IBA and naphthalene acetic acid (NAA) in two MS strengths were capable of inducing roots in the regenerated shoots. The rooting response varied with basal media strength and auxin concentrations. Frequency of root formation was different in all the media. The best root formation response was obtained in ½MS medium containing 2.0 mg/l IBA (Table 3). At this concentration, 75% shoots developed roots which were recorded after 30 days of inoculation with 8.25 ± 0.64 roots per shoot. IBA at a lower concentration responded better than NAA. A number of workers used IBA at lower concentrations for root induction. Baksha et al. (2002), Khatun et al. (2003) and Sabaz et al. (2008) also reported the same. Half strength MS media was most effective to produce roots as reported by Khadiga et al. (2009).

The maximum number of roots was developed in half strength MS + 2 mg/l IBA medium as mentioned in the foregoing. In full strength MS medium, the maximum number of roots (5.35 ± 0.63) was observed at 1.5 mg/l IBA. When NAA were added for root induction, the number of roots recorded was 6.50 ± 0.56 at 2 mg/l concentration in ½ MS medium with 70% response. In full strength, the response was 55% and the number of roots developed was 4.31 ± 0.63 at 1.5 mg/l concentration. A total of 377 plantlets were hardened and transferred to the field and were inoculated with the pathotype isolated from the donor (BF-162).

Screening of red rot resistant somaclones in R_0 generation

The inoculated stalks were cut open longitudinally during the month of September. In the susceptible stalks, the internal tissue showed reddish patches or lesions inter spread with white horizontal patches. As the disease keeps spreading, the internal tissues became dark in colour and dry, resulting in longitudinal pith cavities. At the final stage, grayish cottony growth of the fungus was seen on these cavities. The fungus was re-isolated successfully from the infected canes and was identified as *Colletotrichum falcatum* which was used to inoculate the plant; thus, Koch's postulate for *Colletotrichum falcatum* was completed.

Table 4. Polymorphism in somaclones and their parent 'BF-162' as revealed through random amplified polymorphic DNA (RAPD).

S/N	Primer	Band size	Total band	Polymorphic band	% Polymorphic	PIC
1	GLA-02	300-600	3	2	66.67	0.38
2	GLA-03	300-600	8	2	25.00	0.19
3	GLA-07	250-600	6	2	33.33	0.02
4	GLA-08	250-600	6	1	16.67	0.06
5	GLA-10	300-500	3	1	33.33	0.33
6	GLA-11	300-600	4	1	25.00	0.12
7	GLA-13	300-600	5	1	20.00	0.10
8	GLA-14	300-700	6	3	50.00	0.22
9	GLA-16	250-650	9	2	22.22	0.24
10	GLA-17	350-650	4	1	25.00	0.14
11	GLA-20	350-600	4	1	25.00	0.17
12	GLC-09	300-500	3	2	66.67	0.28
13	GLC-10	300-600	4	2	50.00	0.22
14	GLC-11	400-550	3	1	33.33	0.04
15	GLC-14	400-650	4	1	25.00	0.24
16	GLC-15	350-500	2	1	50.00	0.45
17	GLC-16	300-500	2	1	50.00	0.12
18	GLC-17	480	1	1	100.00	0.22
19	GLC-19	350-600	4	1	25.00	0.19
20	GLC-20	300-600	2	1	50.00	0.43
	Total		83(~4.21)	28		

Only those somaclones in which the pathogen did not cross the nodes adjacent to the point where it was injected were reported to be resistant. Results show that out of the 377 somaclones of genotype BF-162 evaluated for resistance to red rot, 28 were found resistant; 76 were moderately resistant, while the remaining 273 showed variable degree of susceptibility, although, the inoculated donor plants were found to be susceptible. The objective of this study was to generate variability and select the somaclones which should be resistant to red rot disease. In the next two (R_1 and R_2) generations, somaclones were inoculated in R_0 generation. All the somaclones under study were found resistant for their reaction to red rot disease. Keeping in view the objective and the availability of seed by each somaclone, 15 somaclones were assessed for the presence of variability.

Molecular studies

Molecular markers are increasingly being used to study the distribution and patterns of genetic diversity in populations. Many different types of molecular markers are available today.

In this study, two types of markers, that is, random amplified polymorphic DNA (RAPD) and simple sequence repeats (SSR), were used to measure the variability present in the 15 somaclones along with their parent generated through somaclonal variation.

RAPD marker polymorphism

A total of 83 DNA fragments (loci) were generated by the 20 primers used to explore 15 somaclones and their parent. Out of the 83, 28 DNA fragments were polymorphic while 55 were monomorphic indicating 33.73% polymorphism (Table 4). The average number of DNA fragments produced by each primer was 4.21. The size of the amplification products ranged from 250 to 700 bp.

The maximum number of bands (9) was produced by the primer GLA-16, while the minimum number (1) was produced by primer GLA-17. Primer GLC-17 depicted maximum (100%) polymorphism followed by primers GLA-02 and GLC-09 with 66.67% polymorphism. Primers GLA-08, GLA-13 and GLA-16 were found to be least polymorphic with 16.67, 20.00 and 22.22% polymorphism respectively. The PIC indicates the effective number of alleles that can be detected per marker in a set of individuals. It is the discriminatory power of the marker. Data depict that the PIC value ranges from 0.02 to 0.45. Maximum PIC value 0.45 was depicted by primer GLC-15 followed by primer GLC-20 with 0.43 PIC value. The lowest PIC (0.02) was recorded by the primer GLA-07 followed by primer GLC-11 having 0.04 PIC value.

Detection of somaclonal variation through RAPD marker has been applied in sugarcane by many workers (Taylor et al., 1995; Saini et al., 2004; Jain et al., 2005; Suprasanna et al., 2006, 2007; Devarumath et al., 2007).

Table 5. Similarity matrix of somaclones and their parent BF-162 as revealed by RAPD marker.

Somaclone	BF-162 -S-01	BF-162 -S-02	BF-162 -S-03	BF-162 -S-04	BF-162 -S-05	BF-162 -S-06	BF-162 -S-07	BF-162 -S-08	BF-162 -S-09	BF-162 -S-10	BF-162 -S-11	BF-162 -S-12	BF-162 -S-13	BF-162 -S-14	BF-162 -S-15	BF-162 -P
BF-162-S-01	1.00															
BF-162-S-02	0.89	1.00														
BF-162-S-03	0.87	0.91	1.00													
BF-162-S-04	0.89	0.85	0.89	1.00												
BF-162-S-05	**0.94**	0.90	0.90	0.92	1.00											
BF-162-S-06	0.88	0.86	0.86	0.88	0.89	1.00										
BF-162-S-07	0.89	0.87	**0.93**	0.91	0.90	0.90	1.00									
BF-162-S-08	0.83	0.83	0.87	0.85	0.86	0.92	0.89	1.00								
BF-162-S-09	0.88	0.90	0.92	0.92	0.89	0.89	0.92	0.90	1.00							
BF-162-S-10	0.81	0.83	0.83	0.85	0.82	0.82	0.85	0.81	0.86	1.00						
BF-162-S-11	0.82	0.78	0.84	0.86	0.83	0.83	0.88	0.82	0.85	0.84	1.00					
BF-162-S-12	0.89	0.87	0.91	0.91	0.90	0.90	0.91	0.89	**0.94**	0.87	0.86	1.00				
BF-162-S-13	0.86	0.84	0.84	0.84	0.87	0.87	0.86	0.84	0.85	0.78	0.85	0.86	1.00			
BF-162-S-14	0.83	0.83	0.89	0.85	0.84	0.84	0.87	0.87	0.90	0.83	0.92	0.91	0.84	1.00		
BF-162-S-15	0.75	**0.71**	0.75	0.79	0.74	0.74	0.79	0.75	0.80	0.75	0.76	0.77	0.78	0.75	1.00	
BF-162-P	0.78	0.80	**0.84**	0.76	**0.81**	0.77	0.80	0.80	0.79	0.72	0.73	0.80	**0.81**	0.78	**0.68**	1.00

Nair et al. (2002) used 25 RAPD primers to detect variability in 28 elite Indian sugarcane varieties, and they observed 63.74% polymorphism. Lal et al. (2008) investigated variability in sugarcane genotypes using 16 RAPD primers and amplified a total of 110 scorable fragments.

Genetic diversity is commonly measured by genetic distance or genetic similarity, which signifies that there are either differences or similarities present at the genetic level (Weir, 1990). The mean genetic similarity among the somaclones was found as 84.46% (Table 5). This may be due to the fact that somaclones had been derived from the common parent and the changes had occurred at limited numbers of loci in the genome.

The greatest similarity (94.00%) was recorded between somaclones BF-162-S-05 and BF-162-S-01, and somaclones BF-162-S-09 and BF-162-S-12, followed by similarity indices of 0.93 between somaclones BF-162-S-07 and BF-162-S-03. Somaclone BF-162-S-15 had the least genetic similarity (0.71) with somaclone BF-162-S-02. Somaclone BF-162-S-03 had the maximum similarity (0.84) with parent BF-162 (P), followed by somaclones BF-162-S-05 and BF-162-S-13 having similarity indices (0.81). Nevertheless, somaclone BF-162-S-15 had the least genetic similarity (0.68) with the parent, which indicates that maximum changes occurred at the chromosomal or gene level in this clone.

A dendrogram was constructed using fifteen somaclones and their parent BF-162 based on the genetic similarity matrix generated from the 83 RAPD loci for all of the 20 primers. The clustering pattern showed that at a similarity index of 0.84, except for the two somaclones (BF-162-S-10 and BF-162-S-15) and the parent BF-162, the remaining somaclones appeared to form a single cluster (Figure 1).

Somaclones BF-162-S-01 and BF-162-S-05 are the most similar somaclones, while the other pair of somaclones that is still similar is somaclones BF-162-S-09 and BF-162-S-12. However, somaclones BF-162-S-15, BF-162-S-10 and the parent could not be categorized as a separate cluster either. Somaclone BF-162-S-15 is the most divergent followed by somaclone BF-162-S-10, when compared to the parent or the other sister somaclones. Clustering and sub clustering in the dendrogram validated the presence of variability at DNA level. Keeping in view the information that there is close genetic relationship among the somaclones, it is suggested that goal oriented breeding programs with the help of

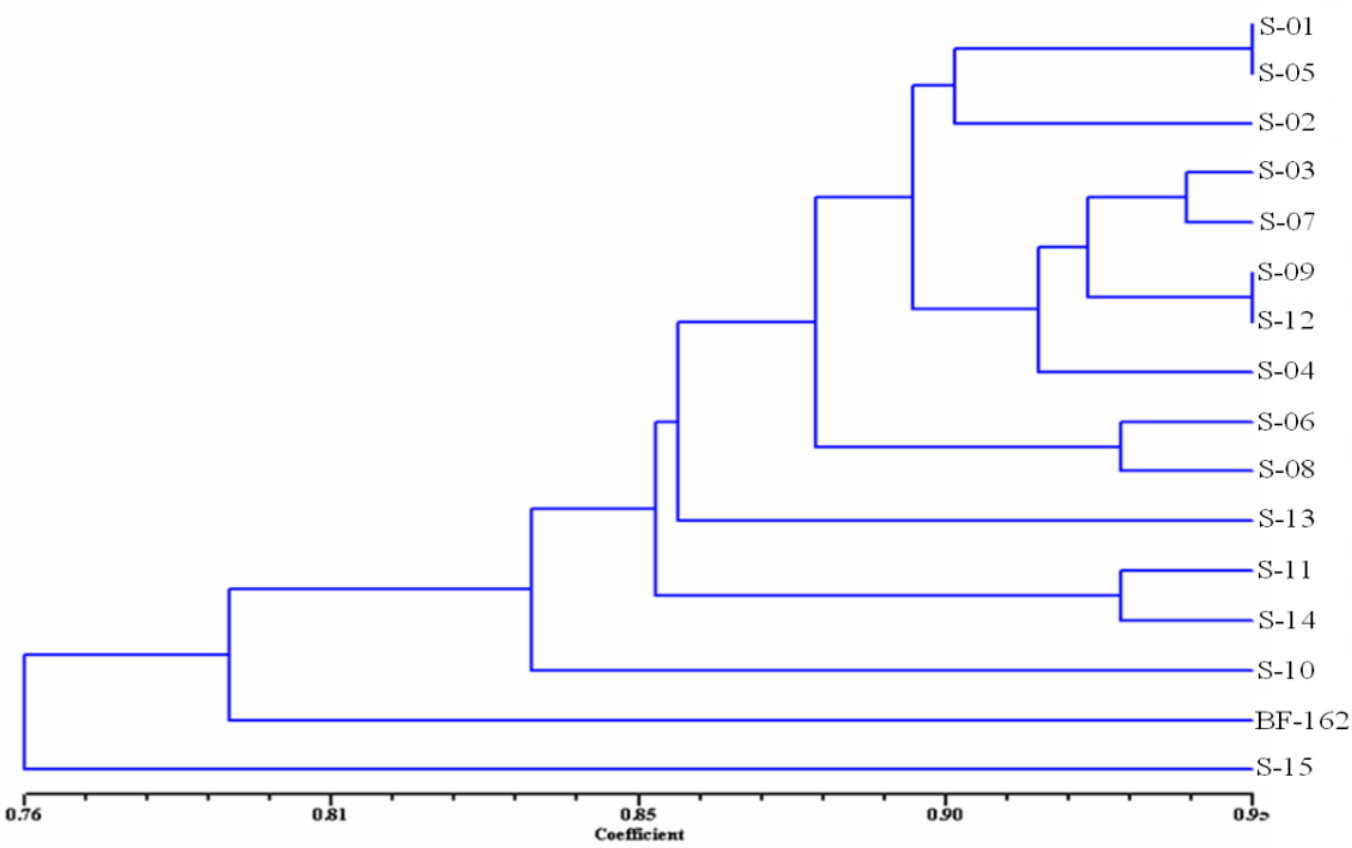

Figure 1. Dendrogram of somaclones and their parent BF-162 as revealed by RAPD marker. S-01 = BF-162-S-01; S-02 = BF-162-S-02; S-03 = BF-162-S-03; S-04 = BF-162-S-04; S-05 = BF-162-S-05; S-06 = BF-162-S-06; S-07 = BF-162-S-07; S-08 = BF-162-S-08; S-09 = BF-162-S-01; S-10 = BF-162-S-10; S-11 = BF-162-S-11; S-12 = BF-162-S-12; S-13 = BF-162-S-13; S-14 = BF-162-S-14; S-15 = BF-162-S-15; BF-162 = Parent.

RAPD technology will be helpful to find out distinct genotypes certainly with diverse genetic background that may improve crop productivity (Rout et al., 1998).

SSR marker polymorphism

SSRs are highly polymorphic and can explore variability even between closely related lines, and are reproducible. Results obtained by the somaclones developed from sugarcane genotype BF-162 are described subsequently. A total of 50 bands were generated by the 28 polymorphic primers used to explore 15 somaclones and their parent. Out of the 50 bands, 32 were polymorphic while 18 were monomorphic showing 64% polymorphism. The average number of bands produced by each primer was 1.78. The size of the amplification products ranged from 100 to 1400 bp. The maximum number of bands (8) was produced by primer SMs006 followed by primers SMs030 and SMs035, which produced 4 bands. The minimum number of band (1), which was polymorphic too, was produced by 17 primers (SSR) with 100% polymorphism. Primer SMs008 was found as least polymorphic (33.33%) followed by primer SMs006 with 37.50% polymorphism.

Jannoo et al. (2001) studied diversity in 96 sugarcane genotypes with just two primer pairs and reported a high level of polymorphism in Mauritius sugarcane germplasm. Cordeiro and Henry (2001) investigated five sugarcane genotypes with 21 primer sets. Among them, 17 pairs were found polymorphic and 0.23 PIC value was reported. Khan et al. (2009) studied the genetic fidelity of direct regenerants of sugarcane with 10 SSR primers. A total of 37 loci were amplified, out of which 30 were found polymorphic, scoring 81% polymorphism. However, the amplified product ranged from 117 to 2191 bp.

The PIC is the measure of the presence of allelic diversity at a given locus. It is the discriminatory power of the marker (primer). Table 6 exhibits that the PIC value ranges from 0.12 to 0.49. Maximum PIC value (0.49) was depicted by SSR primer SMs041 followed by primer SMs035 with 0.45 PIC value. The lowest PIC (0.12) was recorded by primer SMs026 followed by primers SMs012 and SMs043 having 0.17 PIC value.

Table 6. Polymorphism in somaclones and their parent 'BF-162' as revealed by simple sequence repeat (SSR).

S/ N	Primer	Band Size	Total band	Polymorphic band	% polymorphic	PIC
1	SMs002	1000	1	1	100.00	0.38
2	SMs004	200	1	1	100.00	0.22
3	SMs006	100-1400	8	3	037.50	0.36
4	SMs008	500-700	3	1	033.33	0.28
5	SMs009	200-300	2	1	050.00	0.22
6	SMs010	500	1	1	100.00	0.30
7	SMs011	400	1	1	100.00	0.22
8	SMs012	200-900	2	1	050.00	0.17
9	SMs013	500	1	1	100.00	0.38
10	SMs015	700	1	1	100.00	0.22
11	SMs016	900	1	1	100.00	0.38
12	SMs018	100	1	1	100.00	0.22
13	SMs021	300	1	1	100.00	0.22
14	SMs023	300	2	1	050.00	0.26
15	SMs026	200-400	2	1	050.00	**0.12**
16	SMs029	900	1	1	100.00	0.22
17	SMs030	300-500	4	2	050.00	0.26
18	SMs032	300-500	2	1	050.00	0.34
19	SMs033	800	1	1	100.00	0.43
20	SMs035	500-700	4	2	050.00	0.45
21	SMs036	500	1	1	100.00	0.38
22	SMs037	800	1	1	100.00	0.22
23	SMs041	700-1000	2	1	050.00	**0.49**
24	SMs042	650	1	1	100.00	0.38
25	SMs043	1200-1400	2	1	050.00	0.17
26	SMs045	400	1	1	100.00	0.43
27	SMs048	500	1	1	100.00	0.22
28	SMs049	300	1	1	100.00	0.38
	Total		50	32		

The mean genetic similarity among the somaclones derived from parent BF-162 is 73.64%, which shows that a large part of the genome is similar (Table 7). This may be due to the fact that the somaclones originated from the same parent.

The greatest similarity (0.98) was recorded between somaclones BF-162-S-01 and BF-162-S-09, followed by similarity indices of 0.95 among somaclones BF-162-S-12 with BF-162-S-02, BF-162-S-05, BF-162-S-08 and BF-162-S-10; BF-162-S-11 with BF-162-S-08 and BF-162-S-10; and BF-162-S-03 with BF-162-S-06. Somaclone BF-162-S-03 had the least genetic similarity (0.29) with somaclone BF-162-S-15, which shows that changes at maximum loci occurred in this somaclone. Somaclones BF-162-S-12 and BF-162-14 had the maximum similarity (0.91) with parent BF-162 (P), while somaclone BF-162-S-15 had the least genetic similarity (0.38) with the parent. These results support the findings as observed by RAPD, and they suggest that RAPD technique may be utilized successfully to access the genetic similarity and dissimilarities, especially in case of crops where sequence information on SSR primers are not available (Khan et al., 2009). Similarity coefficient matrix were calculated according to Nei and Li's (1979) method used to estimate the genetic divergence and relatedness among the 15 somaclones developed through direct regeneration, ranging from 0.366 (P-100 vs P-98) to 0.951 (parent vs P-104).

A dendrogram was also constructed using 15 somaclones and their parent BF-162 (Figure 2) based on the genetic similarity matrix generated from the 50 SSR loci explored by all of the 28 primers. The clustering pattern showed that at a similarity index of 0.70, three clusters were formed.

Table 7. Similarity matrix of somaclones and their parent BF-162 as revealed by SSR marker.

Somaclone	BF-162 -S-01	BF-162 -S-02	BF-162 -S-03	BF-162 -S-04	BF-162 -S-05	BF-162 -S-06	BF-162 -S-07	BF-162 -S-08	BF-162 -S-09	BF-162 -S-10	BF-162 -S-11	BF-162 -S-12	BF-162 -S-13	BF-162 -S-14	BF-162 -S-15	BF-162 -P
BF-162-S-01	1.00															
BF-162-S-02	0.76	1.00														
BF-162-S-03	0.71	0.86	1.00													
BF-162-S-04	0.81	0.57	0.52	1.00												
BF-162-S-05	0.76	0.91	0.86	0.67	1.00											
BF-162-S-06	0.76	0.91	**0.95**	0.57	0.91	1.00										
BF-162-S-07	0.62	0.76	0.91	0.52	0.76	0.86	1.00									
BF-162-S-08	0.76	0.91	0.86	0.71	0.91	0.91	0.86	1.00								
BF-162-S-09	**0.98**	0.76	0.71	0.81	0.76	0.76	0.62	0.76	1.00							
BF-162-S-10	0.76	0.91	0.86	0.57	0.91	0.91	0.76	0.91	0.76	1.00						
BF-162-S-11	0.71	0.86	0.81	0.52	0.86	0.86	0.81	**0.95**	0.71	0.95	1.00					
BF-162-S-12	0.81	0.95	0.91	0.62	**0.95**	**0.95**	0.81	**0.95**	0.81	**0.95**	0.91	1.00				
BF-162-S-13	0.48	0.62	0.57	0.57	0.71	0.61	0.67	0.71	0.48	0.62	0.67	0.67	1.00			
BF-162-S-14	0.71	0.86	0.81	0.52	0.86	0.85	0.71	0.86	0.71	0.85	0.81	0.91	0.67	1.00		
BF-162-S-15	0.57	0.43	**0.29**	0.76	0.43	0.33	0.38	0.43	0.57	0.33	0.38	0.38	0.71	0.38	1.00	
BF-162-P	0.71	0.86	0.81	0.52	0.86	0.86	0.71	0.86	0.71	0.86	0.81	**0.91**	0.67	**0.91**	**0.38**	1.00

Cluster 1 consisted of 3 somaclones (BF-162-S-01, BF-162-S-09 and BF-162-S-04); Cluster 2 has the maximum number of somaclones (BF-162-S-02, BF-162-S-12, BF-162-S-05, BF-162-S-08, BF-162-S-11, BF-162-S-10 BF-162-S-14, BF-162(P), BF-162-S-03, BF-162-S-06 and BF-162-S-07; and Cluster 3 has only 2 somaclones (BF- 162-S-13 and BF-162-S-15). Somaclones BF-162-S-01 and BF-162-S-09 were found to be the most similar one, whereas somaclones BF-162-S-13 and BF-162-S-15 were seen as the most divergent pair. Formation of clustering and sub clustering in the tree confirmed the presence of variability at DNA level among somaclones with respect to their parent. It verified the reliability of SSR markers to assess the variability present in the somaclone belonging to the same parent as well as confirmed the somaclonal variation as a tool to generate variability in a stable genotype; moreover, the SSR analysis was found as a valuable DNA marker system to evaluate genetic diversity. Nevertheless, it is also suggested that to get more precise results, the number of primers to be surveyed should be increased.

The information generated in this experiment confirmed the capability of SSR marker to determine and estimate the genetic similarity and dissimilarity present among different sugarcane somaclones as well as the reliability of somaclonal variation to generate variability in a stable genotype. The genetic similarity indices may be helpful in forming a population of genetically uniform somaclones. SSR analysis may be very useful also in early identification of most diverse clones present in a population of sugarcane.

This study has identified the usefulness of SSR markers to find out the diversity among somaclones along with their parent. Very limited reports on the use of DNA markers for the estimation of somaclonal variation are available; hence, this study can be used as a point of reference for further studies.

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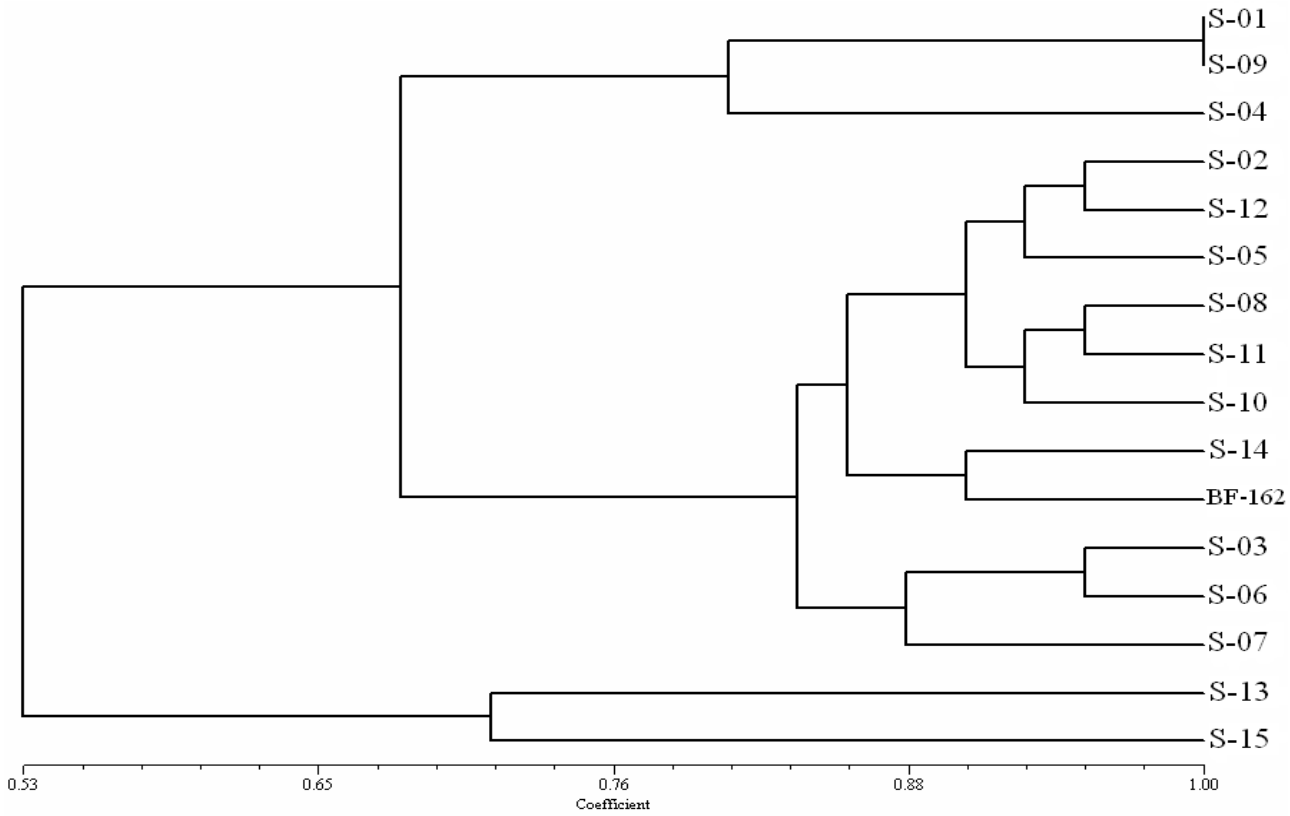

Figure 2. Dendrogram of somaclones and their parent BF-162 as revealed by SSR marker. S-01 = BF-162-S-01; S-02 = BF-162-S-02; S-03 = BF-162-S-03; S-04 = BF-162-S-04; S-05 = BF-162-S-05; S-06 = BF-162-S-06; S-07 = BF-162-S-07; S-08 = BF-162-S-08; S-09 = BF-162-S-01; S-10 = BF-162-S-10; S-11 = BF-162-S-11; S-12 = BF-162-S-12; S-13 = BF-162-S-13; S-14 = BF-162-S-14; S-15 = BF-162-S-15; BF-162 = Parent.

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